The noisy underwater world : the effect of sound on behaviour of captive zebrafish

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Chapter 5

Particle motion and sound pressure in fish tanks: a behavioural exploration of acoustic sensitivity in the zebrafish

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Abstract

There is a growing need to understand fundamental aspects of acoustic sensitivity of fish in both indoor and outdoor conditions. Many fish are kept in fish tanks for aquaculture, hobby, or for biomedical or behavioural research. These tanks can be noisy as surrounding sounds transmit easily into the water via concrete connections between the floor and the tank walls. Fish in natural water bodies are also exposed to elevated levels of anthropogenic noise at an increasing scale worldwide. Underwater sound fields can be complex, especially in fish tanks and in shallow waters, close to surface, rock or bottom. Furthermore, fish are sensitive to both particle motion and sound pressure. We here measured 1) spatial variation in artificially elevated sound levels in a relatively small fish tank, for both particle motion and sound pressure. We confirmed considerable variation over a dynamic range of 25 dB for both components and upward shifts in this range of about 10 dB when close to the tank walls or the bottom and downward shifts of about 10 dB when close to the surface. We also tested 2) whether acoustic response tendency of adult zebrafish (*Danio rerio*) correlated to the sound field conditions at their position at the moment of sound on-set. We found no correlation between the intensity, quality, or directionality of the behavioural response and the sound pressure or directivity and elipticity of particle motion. There was a negative correlation, however, between the tendency to freeze and the average particle velocity level. We suggest that our data provide a basis to further explore the acoustic world of fish in complex environments and may contribute to the study of potential welfare and conservation issues related to anthropogenic noise.

Keywords: captive behavior; experimental exposure; fish welfare; noise impact; sound measurement

Introduction

Ship traffic, wind turbines, pile driving, and seismic exploration can represent a significant component of the underwater soundscapes worldwide. As all fish are capable of detecting sound, acoustic signals and environmental cues play an important role for many fish species in the context of reproduction, orientation and predator-prey interactions (Popper & Fay 1993; Slabbekoorn et al. 2010). The sound characteristics of human activities are typically broadband, more or less temporally structured, and biased towards relatively low frequencies. There is often high structural similarity with biologically relevant sounds and large spectral overlap with the auditory sensitivity of fish. As anthropogenic sounds can be loud and propagate well through water, there is a growing concern about potentially detrimental effects and an increasing awareness about a general gap in fundamental insights about the acoustic world of fish.

To examine the acoustic world of fish and to gain understanding about the potential effects of anthropogenic noise on fishes, both outdoor and indoor experiments are employed. While outdoor experiments provide a high degree of behavioral and acoustic validity, they can be challenging to implement and have a low degree of controllability. Contrastingly, indoor experiments provide a high degree of control but suffer from a lack of acoustic and behavioral validity when compared to open water conditions (Slabbekoorn 2016). While the acoustic differences between natural water bodies and relatively small tanks have been widely acknowledged (Kaatz & Lobel 2001; Parvulescu 1967), there remains a paucity of literature examining these differences from an empirical perspective (Akamatsu et al. 2002; Kaatz & Lobel 2001). Many fish spend time in shallow waters or in close proximity to surface, rock, or bottom boundaries, where the sound fields are more complex than in far field, open water conditions. Furthermore, captive fish just experience artificial sound fields in fish tanks that can be unintentionally or experimentally noisy.

Fish can hear both the pressure and particle motion components of acoustic waves. All fish are able to detect acoustic particle motion using a specialized structure called the otolith organ within the inner ear, which is able to extract frequency and amplitude information from oscillating motions, analogous to an accelerometer (Fay 1984). Fishes possessing a swim bladder are also able to detect the pressure component of sound through pressure-to-motion conversion via the air-filled cavity of the swim bladder, which expands and contracts in response to pressure changes (Popper & Fay 2011). Specialized adaptations like the Weberian apparatus in Ostariophysians can further enhance the acoustic sensitivity to sound pressure by acting as an efficient conduit for kinetic energy between the swim bladder and the inner ear. These specialized adaptations can increase both the frequency range and absolute hearing thresholds (e.g. Schulz-Mirbach et al. 2012).

Many studies have assessed hearing thresholds and acoustic response tendencies in fish (Popper & Fay 1973; Horodysky et al. 2008). Many of these studies are done in laboratory facilities and with the fish close to the surface in a small tank which complicates the interpretation and comparison of results. It is therefore wise to treat absolute acoustic measures from such studies as study-specific and not as general truth. However, relative sensitivity information across the spectrum should also be treated with care, as this involves the outcome of overlapping ranges of perception through both particle motion and pressure, for which the sound field conditions are highly variable with dynamic ratios between the two components under typical indoor fish tank conditions (Parvulescu 1967; Rogers & Cox 1988). Some studies have compared fish hearing thresholds for particle motion and pressure by isolating these acoustic components within the experimental setup, exposing fish to acoustic signals comprised exclusively of either particle motion or pressure (Bretschneider et al. 2013; Wysocki et al. 2009). Although these studies revealed some more advanced insights into fish auditory perception, there remains especially little knowledge regarding how fish react behaviourally when exposed to variable ratios of the two components.

Although many fish do not reside in far field, open water sound conditions, this is still a useful reference for exploring more complex sound fields. In far field, open water conditions, a propagating sound shares a fixed relationship between its sound pressure and particle motion components, thus the predicted far-field particle velocity (PFV) for a given sound pressure measurement is calculated using Eqn 1:

$$PFV = \left(\frac{rms(p_{measured})}{c \cdot \rho}\right),$$

(1)

where $rms(p_{measured})$ is the root mean square of the measured sound pressure over time (µPa), c is the speed of sound in water (1482 m/s), ρ is the density of water (1027 kg/m^3), and the resulting PFV is returned in µm/s.

While the relationship between sound pressure and particle motion under these conditions is generally constant, most small tank experiments are conducted in the acoustic near field due to the low frequencies of interest and relatively small dimensions of the tanks used. In the near field, sound radiates in a spherical pattern, resulting in relatively higher levels of particle motion closer to the sound source (Bretschneider et al. 2013), as compared to far-field conditions.

A critical parameter of the sound field to understand behavioural response patterns is the directionality of the particle motion (Schuijf 1975; Van den Berg & Schuijff 1985; Popper & Fay 1993; Rollo & Higgs 2008). In a boundless far field environment with a single sound source, the directionality is observed as a one-dimensional oscillation of particles along the axis parallel to the direction of the propagating wave. However, under spatially restricted conditions such as small tanks, fish are continuously exposed to reflected sound waves. When two sound waves arriving from different directions propagate through a common point, the particle motion components of both waves will combine following the rules of vector addition. Additionally, the phase difference resulting from the latency in travel times between the incident and reflected waves can cause a two- or three-dimensional oscillation of particles which can be characterized by particle ellipticity.

Current models of fish hearing are based on the assumption that fish determine the direction of sound propagation through acoustically induced otolith motion along the axis of the acoustic wave (Rollo & Higgs 2008). As points in an acoustic field with high particle ellipticity will result in otolith motion that deviates from a single axis of displacement, this suggests that particle ellipticity may undermine or contribute to the ability of fish to localize sounds by convoluting the directional component of otolith motion. To our knowledge, there is currently no literature describing particle ellipticity within the context of sound source localization by fish.

In this study, we conducted two experiments in relatively small tanks: one in which we measured particle motion and sound pressure levels to explore the relationship between the two sound components and a second to explore the potential relevance to fish. The first experiment examined how the ratio of sound pressure to particle motion in a small tank varies in response to the spatial location within the tank, as compared to theoretical open-water conditions. In the second experiment, we further examined the sound pressure and particle motion components within the context of an acoustically induced behavioural response experiment using zebrafish (*Danio rerio*). We compared the 1) occurrence, 2) intensity, and 3) direction of acoustically elicited startle/fleeing responses for individual fish to the predicted sound pressure and particle motion conditions they would have experienced at their location during the on-set of sound exposure.

Methods

Experiment 1

Experimental Setup

The experimental tank used in the present study was constructed from glass and had the following dimensions: 100 x 50 x 50cm, a wall thickness of 0.75cm, and a water depth of 40cm. The tank was positioned on a table on top of ~4cm of acoustic insulating material to reduce acoustic artifacts caused by building vibrations. Within the tank, the acoustic field was measured along a three-dimensional grid at 10cm increments using a custom-built vector sensor (c.f. Bretschneider 2013; Shafiei Sabet et al. 2015). The vector sensor was positioned along this grid using two perpendicularly oriented red lasers (λ = 635nm in air). The Perspex sphere containing the three accelerometers was hanging in the water by two nylon wires that allowed position control due to the slightly negative buoyancy of the sphere. This system allowed us to position the vector sensor within a

~1cm range of accuracy. All measured positions in this grid were at least 10cm away from the tank walls.

The tank was ensonified using a JBL EON500 in-air speaker (USA, Maximum volume, Equalizer: Boost) connected to a DR-05 handheld recorder (Tascam, USA) at a distance of 1.5m with the speaker facing the center of one of the two widest walls of the tank. During each acoustic measurement, the experimental tank was ensonified with 10 seconds of white noise. The white noise playback track was artificially generated in Audacity (http://audacityteam.org/, version 2.0.5) and a bandpass filter was applied between the frequency ranges of 100-1000 Hz. The playback volume of the in-air speaker was adjusted so that a sound pressure level (SPL) of 112dB (re 1 μPa) was measured in the center of the tank with a calibrated HTI 96-min hydrophone (High Tech, USA) connected to a DR-100MKII recorder (Tascam, USA).

In addition, a supplementary set of measurements was taken to investigate the effect of changing speaker volume where the vector sensor was placed in the vertical center of the tank, 14cm away from the wall closest to the speaker. The tank was then ensonified with the same white noise exposure 21 consecutive times, with each exposure digitally set to be 2dB quieter than the previous.

Acoustic Measurements

All sound pressure and particle motion measurements were recorded with the custom-built vector sensor and amplifier that was previously used in studies by Bretschneider et al. (2013) and Shafiei Sabet et al. (2015). This was then connected to a Picoscope 3425 USB Oscilloscope (Pico Technology, England & Wales) and data was logged from the oscilloscope using a program written in Visual Basic for Applications within Microsoft Access 2010 (Microsoft, USA).

The vector sensor was calibrated in reference to a pre-calibrated M20 directional hydrophone (Geospectrum Technologies Inc., Canada). The calibration was conducted by suspending the M20 directional hydrophone in the center of the large tank and ensonifying the tank from an in-air speaker 1.5 m away. The M20 directional hydrophone was then replaced by the custom-built vector sensor and the exposure was repeated. By comparing the resulting measurements from the two devices in the frequency domain, we were able to construct a receiver sensitivity graph for each channel of the custom-built vector sensor. As the acoustic environment in the experimental tank is prone to artifacts and the differing size of the sensors results in unequal sampling areas, a degree of inaccuracy is to be expected from this calibration method. Frequency ranges within the resulting receiver sensitivity graph that appeared to be inconsistent over repeated calibrations were discarded, resulting in a final calibrated range of 50-1000Hz.

Acoustic Analysis

All audio analyses were conducted using Matlab (Mathworks, USA, Version 8.1) with a bandpass filter applied between 100-1000Hz (the calibrated range of our vector sensor) and following the standardized definitions for each measurement as seen in Ainslie (2011), unless otherwise specified. Particle velocity measurements were reported as sound velocity level (SVL), and are defined according to Eqn 2:

$$SVL = 20 \cdot log_{10}\left(\frac{rms(u_{measured})}{u_{reference}}\right) \text{ dB} \tag{2}$$

where $rms(u_{measured})$ is the measured root mean square of the particle velocity over time and $u_{reference}$ is the reference particle velocity (1nm/s).

To compare SVL and SPL measurements in a context relevant to open water experiments, we examined the excess SVL. This measurement was calculated by subtracting the expected SVL under far field, open water conditions from the measured SVL in the tank as shown in Eqn 3:

$$Excess\ SVL = 20 \cdot log_{10}\left(\frac{rms(u_{measured})}{EFV}\right) \text{ dB.} \tag{3}$$

Under far-field open water conditions, SPL is expected to show no relationship with excess SVL, and as a result, excess SVL measurements taken in these conditions would be expected to be 0dB. Excess SVL measurements taken close to a sound source are expected to be higher than those taken further away due to near field effects of spherical sound propagation.

Statistical Analysis

All statistical analysis were carried out in R (version 3.2.2, including the packages: ggplot2, nlme, lme4, MASS, CircStats). We examined the relationship between the spatial parameters (i.e. the position of the vector sensor in the tank) of each acoustic measurement and the resulting SPL and SVL values in the experimental tank using Generalized Linear Models assuming a Gaussian error distribution. The selection of variables used in each model was determined by AIC stepwise selection (both directions). The spatial variables included in the model selection were the continuous variables: distance from the tank wall closest to the in-air speaker, distance from the closest tank wall facing the direction adjacent to sound propagation (including the second degree orthogonal polynomial), distance from the bottom of the tank and the binomial variables: close to tank bottom or water surface and close to either wall facing the direction of sound propagation. A visual examination of the residual plots for each model indicated that there were no significant deviations from the assumptions of normally distributed residuals.

For examining the relationship between Excess SVL and the spatial variables, we again used a Generalized Linear Model with assumed Gaussian error distribution. The variables used for the model selection are the same as used in the SVL/SPL comparison, except for the addition of SPL as a fixed effect and the use of Excess SVL as the responding variable.

Experiment 2

Experimental Setup

The behavioural response experiment was conducted in the same in-air speaker tank setup as in experiment 1, with the exceptions that the speaker was placed 1m away, instead of 1.5m, and a restricted swimming area measuring 24cm x 10cm x 10 cm was placed within the glass tank to constrain the fish to a small area where we had measured highly variable particle motion to sound pressure ratios (Fig. 1).

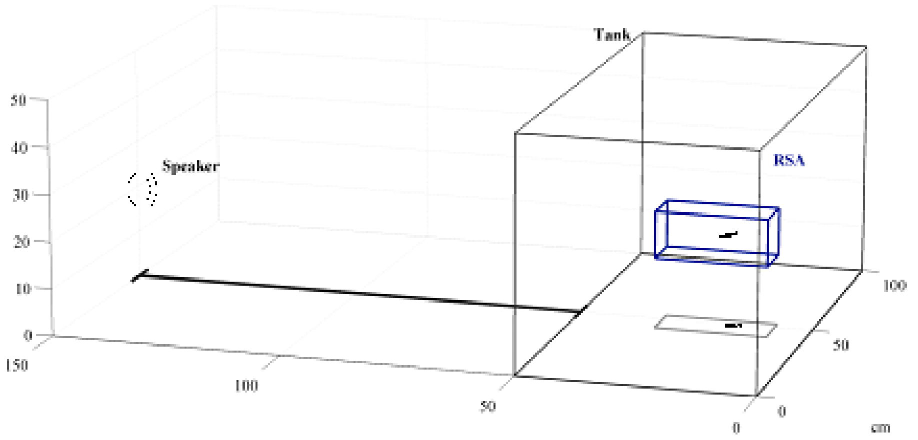


Fig. 1. Scaled 3D image of the setup used in the behavioural response study of Experiment 2. The acoustically and visually transparent restricted swimming area is labeled “RSA” and highlighted in blue.

The restricted swimming area was constructed from a rectangular iron frame with walls made of plastic wrap. Plastic wrap was chosen because of its visual and acoustic transparency. During the pilot trial, a comparison of measurements taken in the same positions both with and without the restricted swimming area surrounding the sensor resulted in no observable difference in SPL or SVL measurements. Two HC-V500 video cameras (Panasonic, Japan) set to record at 50 fps (interlaced) were placed above and to the side of the tank to obtain a dorsal and lateral view of the startles and distinct fast start responses Mirjany et al. 2011; Domenici & Blake 1997). The volume level of the DR-05 handheld recorder attached to the EOS500 loudspeaker (Maximum volume, Equalizer: Flat) was adjusted in this behavioural experiment to achieve a SPL of 120dB in the center of the tank. Playback tracks used in this experiment consisted of a one hour period of silence followed by 10 one-second pulses (white noise, 10-2000Hz) randomly distributed over a three hour period. The random placement of the pulse noises was determined by dividing the 3 hour trial period into 10 segments of 18 minutes. A pulse was then played at a randomly selected minute within each 18 minute segment.

Once the water was warmed to at least 22°C, the trials began by placing an individual into the restricted swimming area within the large tank and the playback track was started after the video cameras had begun recording. The start and end temperatures were recorded for 12 of the 14 trials and tank heaters were removed during the trials. Temperatures ranged from 22.5-24°C upon the start of each trial and the maximum drop in temperature by the end of a trial was 1.5°C. In addition, the room hosting the experiment had no windows, thus lighting conditions could be kept consistent throughout all the trials. A LUNASIX F light meter (P. Gossen & co, Erlangen, Germany) was used to measure the experimental light conditions by placing the light meter 5 cm above the water surface in the horizontal center of the tank, resulting in a light illuminance of 1290 lux. Upon the start of the playback track, we left the room and did not return until after the 4 hour trial period had ended. Because of moderate but regular background noise and vibrations due to nearby building maintenance during the morning and early afternoon, all trials were initiated between 15:45-16:40 and we only conducted one complete trial per day (one fish per day).

Behavioural Analyses

Each trial had a unique timing pattern for sound exposures and we assessed the spatial position of the fish at each pulse moment in the trial sequence. For each sound exposure, one minute of video before and after the onset of each pulse was extracted for analysis and converted to a Motion-JPEG video format (50 frames per seconds, progressive scan) using FFmpeg (https://www.ffmpeg.org/, version 2.4). Location tracking of the individuals was then conducted in Matlab using a background subtraction algorithm based on brightness values. We reviewed all video analysis data and we manually corrected tracking errors. We combined the information from the dorsal and lateral cameras to provide three-dimensional locational data for all sound exposures.

We used the video recordings to score behavioural states related to swimming speed: startle and fast start onset and freezing. The presence of distinct startles and onset of the fast start responses were defined by any sudden quick movement which followed the first and second stage motions associated with fast start responses in zebrafish (Mirjany et al. 2011). Freezing was defined by the lack of swimming activity or interruption of all activities except breathing (c.f. Shafiei Sabet et al. 2016). We scanned for fast start responses within 100 frames (2 seconds) before and after the onset of the sound exposure. In circumstances where a fast start response was suspected but not obvious to the observer, these were treated as expressing no fast start response. The sound conditions of each potential response were determined independently and after behavioural assessments and the scoring by the observer can thus be regarded blind to the treatment.

To collect more precise directional information during the startle response, the midline of the individual was traced by hand over a period of 1 second before and after the startle response. The midline was defined as a straight line drawn from the snout of the fish to the midpoint between the pectoral fins (Mirjany et al. 2011). Because of the low temporal resolution of the video footage and the relative quickness of startle reposes, the midlines could not be quantified accurately in three-dimensional space. Consequently, only the camera positioned above the tank was used to analyze the directional component of the startle responses.

Quantifying the Acoustic Field at Startle Response Locations

The acoustic field in the restricted swimming area was measured with the same calibrated vector sensor as used in experiment 1. The area enclosed by the restricted swimming cage was measured following a two-dimensional grid along 5cm increments at the center depth of the restricted swimming area (20cm). To predict the sound field characteristics of SPL, SVL, and the direction of particle motion at the exact locations of the startle responses, the grid data function in Matlab was used to conduct two dimensional linear interpolation on the measured acoustic field values (See Fig 4). Due to the flexible nature of the plastic wrap walls and the small degree of error in the video tracking, when the fish was close to the walls of the restricted swimming area during the onset of noise exposure, some interpolation points resided outside of the measured sound field and could not be interpolated. These points were excluded from the analysis.

To calculate particle ellipticity, the paired measurements of particle velocities for the X and Y channels of the vector sensor were plotted in a bivariate histogram (Fig. 2). A convex hull was then drawn around all values which were >25% of the maximum frequency in the histogram. Particle ellipticity was then calculated by comparing the length of the major axis of the convex hull to its adjacent axis using Eqn 4:

$$Particle\ Ellipticity = arctan(\frac{l_{minor}}{l_{major}}) \cdot (\frac{180}{\pi}),$$

(4)

where l_{minor} and l_{major} are the lengths of the major and adjacent axes of the convex hull, respectively, and the particle ellipticity is returned in degrees. Linear interpolation was again used to predict the particle ellipticity values at the exact location of the fish during the onset of noise exposure.

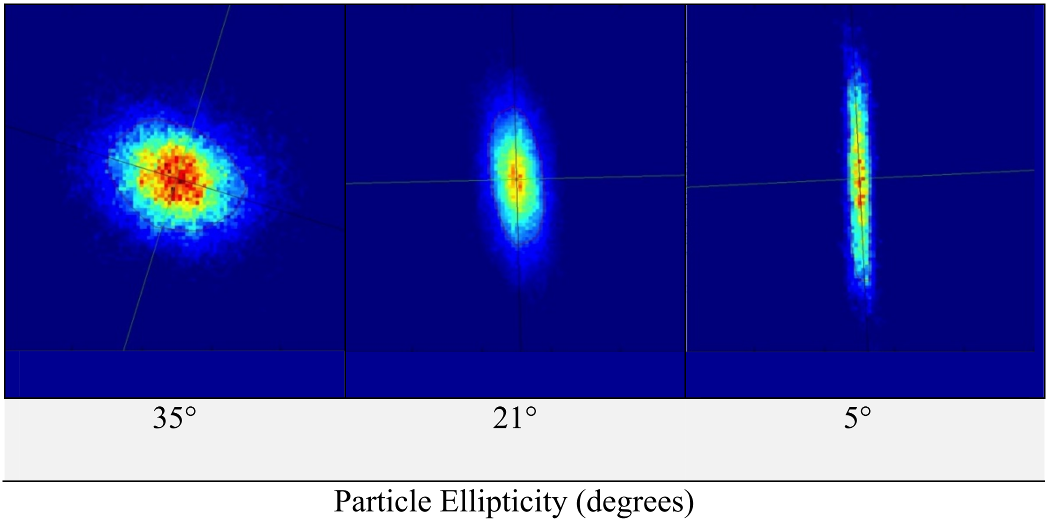


Fig. 2. Bivariate histograms of the instantaneous particle velocity along the x and y channels of the vector sensor over a period of 4 seconds during playback of white noise, band-pass filtered between 50-1000Hz. The center of each image is 0m/s for each channel and the particle ellipticity is reported in degrees. The black line represents the major axis of particle velocity while the green line represents the axis perpendicular to this major axis. A value of 45° indicates perfectly circular particle motion (the particle velocity measured along the major and adjacent axes are equal), while smaller values represent increasingly linear particle velocity.

Statistical Analysis

The effect of sound field components on the intensity of startle responses was examined with a Linear Mixed Effects Model (maximum Likelihood method) with a Gaussian Error distribution to predict the post-exposure average swimming speed and a Generalized Linear Effects Model with a Binomial error distribution to predict the probability of a freezing response within 50 seconds after the exposure. A visual check of residual plots was used to confirm that the assumptions of normally distributed residuals were met. In both models, the individual was defined as the random effect (random intercept) and the average swimming speed was calculated over a period of 10 seconds before and after the onset of noise exposure.

We determined the inclusion of the following fixed effects by AIC-score: SVL at the fish's location during the onset of noise exposure, SPL at the fish's location during the onset of noise exposure, and the average swimming speed before the onset of noise exposure. A linear regression analysis was used to explore collinearity between the paired SVL and SPL estimates, but the relationship was not significant. The fixed effect expression of freezing behavior before the onset of noise exposure was also included in model construction to distinguish between cases in which the fish was swimming normally prior to the sound exposure and then froze in response to it, as opposed to a false detection when the fish was already frozen before the exposure and remained frozen during and after the exposure.

Predicted SVL and SPL values at the individual's location during the onset of noise exposure were also compared to the occurrence of startle responses and the change in post-exposure swimming speed, but no correlations were evident. The final mixed effects models only included exposures that resulted in visible startle responses and the marginal and conditional R^2 values for each model were calculated according to

Nakagawa & Schielzeth (2013), where the marginal R^2 represents the variance explained exclusively by the fixed effects and the conditional R^2 represents the variance explained by both the fixed and random effects.

Circular statistics were employed to examine if there was a directional response related to the sound-field properties during the startle responses. The direction of escape during the fast start response over the temporal scales of 1,2,3,4, and 5 frames (Each frame is spaced 20ms apart) after an observed response was compared to the direction of particle motion analyzed over the bandwidths of 50-150Hz, 150-250Hz, 350-450Hz, and 750-850Hz. Because the mechanism which fish use to determine the acoustic directionality of particle motion is poorly understood, we treated the direction of escape as a diametrically bimodal distribution in which a value of 0 radians represents the fish swimming in either direction parallel to that of acoustic particle motion and a value of π radians as a direction perpendicular to that of particle motion.

Ethical approval

A total of 15 zebrafish were used in the experiment, one of which was exclusively used for a pilot trial and excluded from the final dataset. All experiments were performed in accordance with the Netherlands Experiments on Animals Act (DEC approval no: 13022) that serves as the implementation of the Directive 86/609/EEC by the Council of the

European Communities regarding the protection of animals used for experimental and other scientific purposes (1986).

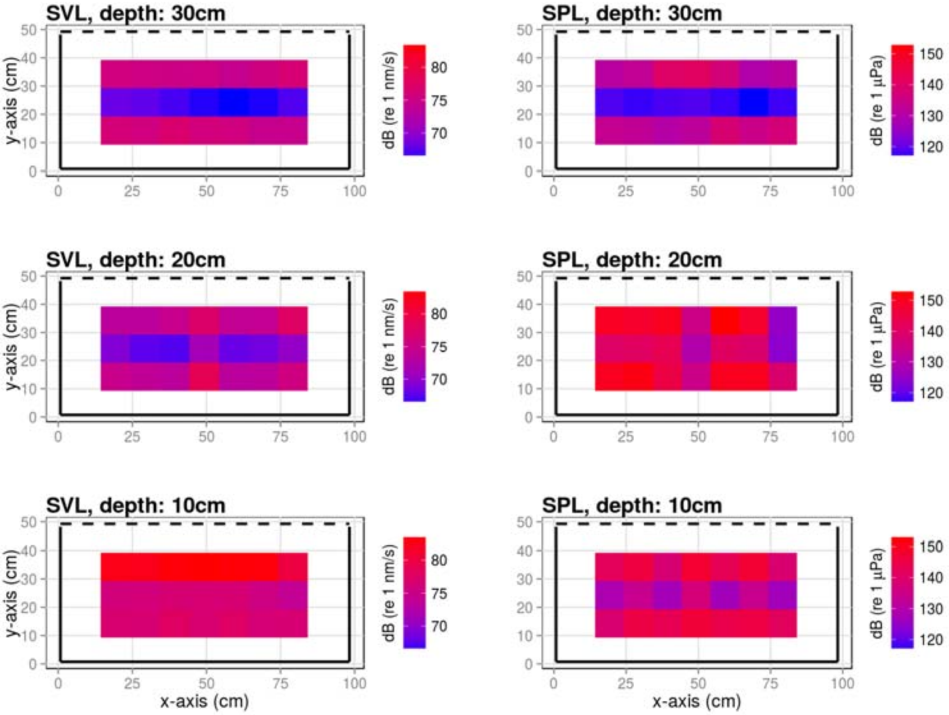
Results

Experiment 1

SVL and SPL

The SVL and SPL components of the measured sound field followed generally similar trends within the tank (Fig. 3). Both varied considerably over a dynamic range up to 15dB for SVL and 25dB for SPL and at any particular distance from the wall nearest the speaker or at any particular depth. Most notably, the sound level ranges in the center of the tank were shifted down approximately 5dB for SVL and 10dB for SPL, as compared to locations close to both tank walls. Similarly, for sound pressure the sound level range was lower for measurements close to the surface relative to in the middle and at the bottom of the water column. SVL ranges were highest at the bottom relative to both the middle and at the top of the water column. There were no significant interaction effects in the SPL model, but we found a highly significant interaction effect in the SVL model between the distance from the wall closest to the in-air speaker and the distance from the bottom of the tank ($T_{53} = -6.98$, $P = 4.86e-9$).

Trends in excess SVL measurements relative to the spatial positions within the tanks were generally similar to those observed in the SVL and SPL measurements, as the excess SVL is calculated from both SVL and SPL. In addition, SPL showed a highly significant negative correlation with excess SVL (Table 1). A supplementary set of measurements taken while the vector sensor was stationary, and the volume of the playback track was adjusted support these results (Fig. S1). Observed Excess SVL values ranged from -15.1 to 16.2 dB across all sampling positions.



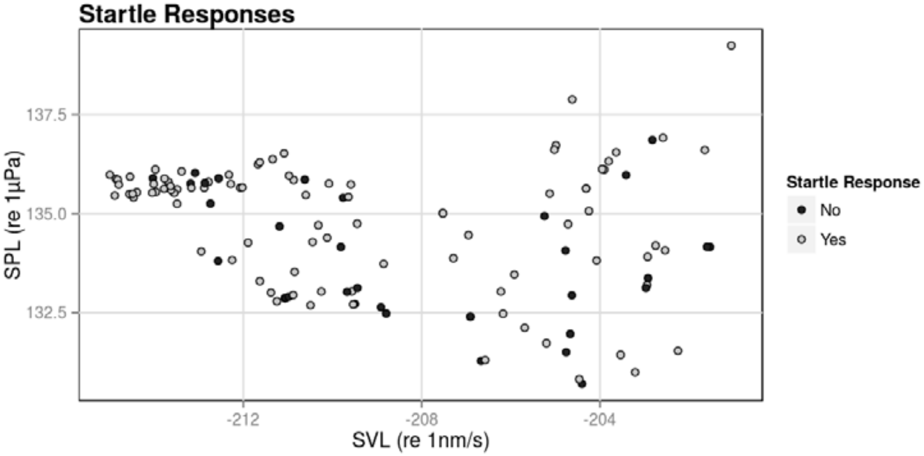


Fig. 3. Spatial variation in sound field conditions in the experimental fish tank. Rasters of the SVL and SPL measurements reflect sound field variation throughout the tank at a 10cm resolution. Black lines represent the four side walls of the tank, with the dotted line representing the wall closest to the in-air speaker.

Fig. 4. Occurrence of distinct startle and/or fast start swimming response (grey dots) and lack of any visible response (black dots) for fish in the restricted swimming area at locations with variable interpolated SVL (dB re 1 (nm/s) and SPL (dB re 1 µPa) measurements, as indicated on the x-axis and y-axis respectively. At higher SVL there is higher variability in associated SPL, but both modalities seem to vary more or less independently. There is no correlation between whether or not there is a startle response and either SVL or SPL measurements.

Experiment 2

The mixed effects model predicting post-exposure swimming speed revealed that the pre-exposure swimming speed, pre-exposure freezing behavior, and exposure number were significantly correlated with a decrease in the change of swimming speed, although a majority of the explained variance was accounted for by the random effect of the individual ($R^2_c - R^2_m = 0.28$). SPL and SVL were not significantly correlated with a change in swimming speed. The analysis results are summarized in table and illustrated in Fig. 5.

The mixed effects model predicting the probability of a freezing response within 50 seconds after noise exposure revealed that higher SVL measurements resulted in a lower probability of a post-exposure freezing response, while SPL showed no relationship. In addition, the average pre-exposure swimming speed was also negatively correlated with the probability of a freeze response. A majority of the variance was accounted for by the random effect of the individual ($R^2_c - R^2_m = 0.47$). The analysis results are summarized in table and illustrated in Fig. 5.

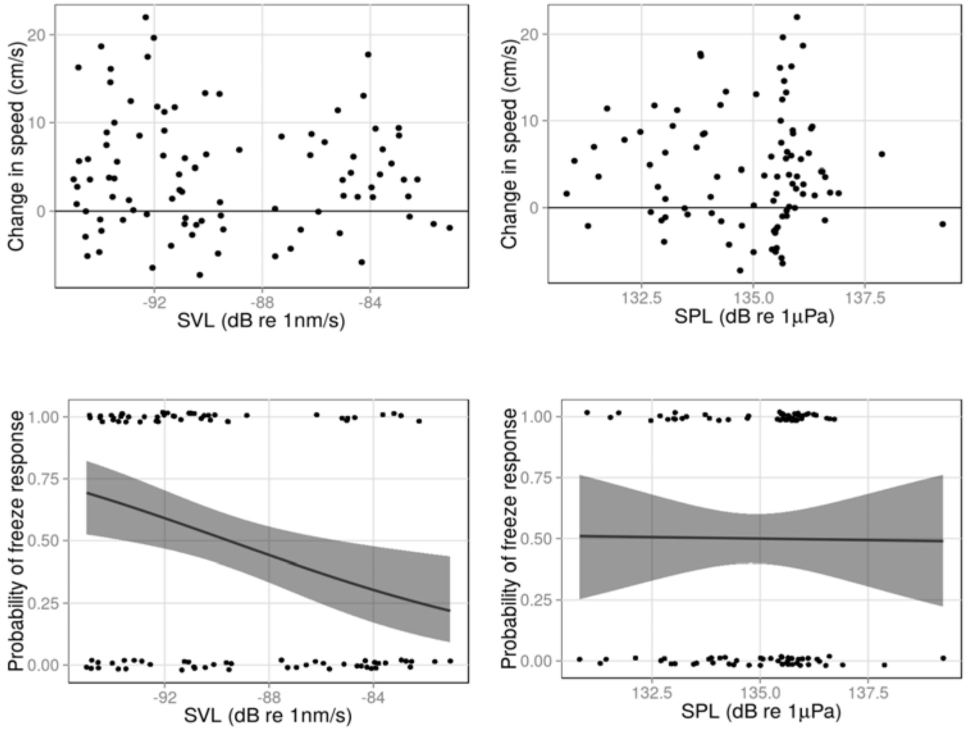


Fig. 5. Interpolated SVL (dB re 1 (nm/s) and SPL (dB re 1 μPa) values at the fast start response locations across all individuals compared to the change in swimming speed averaged over 10 seconds before and after noise exposure (top) and the probability of a freeze response within the 50 seconds after noise exposure (bottom). Y-axis variability has been added to the points on the bottom plots in addition to a LOESS curve with 95% confidence interval as a visual aid. Mixed effects models revealed that the probability of a freeze response was negatively correlated with SVL (bottom left).

Rayleigh's test (mean direction alternate hypothesis) and Watsons test of uniformity showed that the direction of escape was not significantly different than that of a uniform circular distribution, except in the temporal range of 5 frames after the first observed startle motion and over a bandwidth of 750-850Hz (Rayleigh's test: mean resultant length = 0.044, p-value = 0.011; Watsons test: $U^2 = 0.182$, p-value < 0.1). A one-tailed binomial test was then done on the non-uniform distribution to determine that there was a significant preference to escape in a direction parallel to that of particle motion ($X^2 = 2.769$, p-value = 0.048). A Watson's two-sample test was further used to check if the resulting distribution fitted a von Mises distribution, but the results were not significant.

Discussion

Our results provide new insights into the sound field complexity of relatively small fish tanks and into the challenging exploration of the link between sound field parameters and fish behaviour. In experiment 1, we showed that the SVL and SPL components of the sound fields within the experimental tank followed generally similar trends with relatively high SVL and SPL close to tank walls and relatively low SVL and SPL close to the surface. Furthermore, the excess SVL deviated well above and below open water, far field conditions, revealing considerable variation throughout the fish tank between SVL and SPL measurements taken at the same position. In experiment 2, we found a similar, highly variable pattern of acoustic measurements at spatial locations of zebrafish in the restricted swimming area, including SVL, SPL, but also sound velocity direction and ellipticity. We also found a general lack of correlations between acoustic and behavioural measurements such as speed and direction of swimming response. However, locations with higher SVL values during noise exposure were correlated with a lower probability of a post-exposure freezing response.

Fish tank acoustics

Our acoustic measurements confirmed that SPL, SVL, and excess SVL in small tanks are highly variable across spatial locations. Both, absolute levels and spatial and temporal variability stray from the theoretical values that are expected to be experienced by fish swimming in open water, far field conditions. Consequently, indoor sound field assessments and behavioural response studies can be valuable to gain fundamental understanding about underwater acoustics and insights into housing conditions of fish in captivity, but they are unlikely to shed much light on free-ranging fish in outdoor conditions. Nevertheless, as mentioned before, many fish occur in natural habitat with more complex sound fields than open water, far-field conditions. Indoor insights can therefore turn out valuable for future explorations of sound impact on fish in shallow waters, close to surface, rock or bottom.

We believe our measurements reveal several interesting findings, some of which expected and others not fully understood yet. The relatively low levels of SPL observed close to the water surface in our tank are in line with expected sound pressure release characteristics of the water-air boundary. However, we also expected relatively high levels of particle motion at the surface and that is not reflected by our measurements. This discrepancy may be caused by additive effects from the four walls and bottom as secondary sound source and the resultant patterns of reflected waves.

We also observed higher SVL and SPL values closer to the bottom and closer to either tank wall, largely independent of the speaker side. This suggests that the whole tank acts as a vibrating rigid body in response to in-air sound waves. This is not surprising as for an acoustic wave to pass from the outside air to the water within the tank, the tank walls must vibrate to transmit the acoustic energy between the two mediums. The vibrations are likely conducted among adjacent tank walls, resulting in the entire tank serving as a secondary sound source. Consequently, from the perspective of a fish within the tank, the sound field is not likely to carry much information about the location of the in-air speaker as the primary sound source.

We did find some acoustic variation in the water along the axis of sound propagation in air. Measurements taken close to both the bottom of the tank and the wall closest to the in air speaker resulted in higher SVL measurements and a significant interaction effect between horizontal and vertical variation. As this interaction effect is only visible very close to the fish tank boundaries and absent for SPL, we expect it may result from the differing area size of sampling between the hydrophone (~1-2 cm diameter) and the geophones mounted within our vector sensor (9.5cm diameter). Due to this size difference, the particle motion component of the vector sensor is sampling about 3.5 cm closer to any given sound source across all locations as compared to the paired samples from the hydrophone.

Exploring acoustic sensitivity of fish

We found our captive zebrafish to respond to sudden sound bursts of moderate levels, like in earlier experiments (Neo et al. 2014; Shafiei Sabet et al. 2015). We succeeded in triggering behavioural responses in many but not in all cases with a variety in SVL and SPL levels and variable combinations thereof. Despite reaching these experimental targets for an optimal test of whether response tendency and intensity are related to particular parameters of the local sound field, we did not find clear correlations between sound parameters and our expected behavioural response patterns.

We did, however, find one significant correlation between sound and behaviour: the probability of a freezing response was negatively correlated with the SVL at the fish's location during sound exposure. However, we believe this is in contrast with any logical expectation. Freezing responses, in concert with thrashing and erratic swimming, has been shown to be a reliable indicator of anxiety in the context of, for example, light conditions or perceived predation risk (e.g. Blaser et al. 2010; Bass & Gerlai 2008; Cachat et al. 2010) and has also been scored as such in earlier sound impact studies with this species (Shafiei Sabet et al. 2016). Consequently, if SVL was perceptually the most prominent of all sound field features and responsible for a correlation via a causal relationship, one would expect a positive correlation: higher levels triggering more freezing.

Although we are not convinced about the causal relationship of SVL and behavioural response tendency in our current study, we do see this finding as a confirmation that our set-up could work. Quantifying additional behavioral metrics, like thrashing and erratic swimming, may provide additional insights about the nature and potential for underlying physiological impact of fast swimming or freezing responses (Bass & Gerlai 2008). Integrating detailed sound field characterization and detailed behavioural assessments of free-swimming fish may yield specific correlations that indicate perceptual prominence for one among multiple audible sound parameters. This appears still quite a challenge, but also perceptual weighting studies on acoustic parameters of song in birds have only become possible after many years of methodological progress in different laboratories (e.g. Dooling & Okanoya 1995; Beckers et al. 2003; Pohl et al. 2012).

Methodological potential and problems

As we hope that our study will stimulate follow-up, we here address some methodological potential and problems in our set-up. First of all, we see potential in our approach with a restricted swimming area to keep the experimental fish in a specific part of an indoor fish tank where variation of particle motion and sound pressure levels are measurable and within certain limits. It should, however, be noted that swimming restrictions, in captivity in general and for further spatial restrictions in particular, also limit natural behavioural response patterns (Calisi & Bentley 2009; Slabbekoorn 2016; Neo et al. In Press). Our analysis of the swimming direction of startle responses, for example, yielded no relationship with the direction of the SVL component of the playback sound, except when examining the fish's location at 100ms after the startle response over a bandwidth of 750-850Hz. This result is inconclusive but potentially due to the small and rectangular shape of our experimental area: fish may have preferred to escape in the direction with the largest free area for movement which would cause a bias in escape directions (also see Shafiei Sabet et al. 2016).

As a second point, we like to draw attention to the potential for using stimuli of variable frequency to study fundamental aspects of hearing. Zebrafish are most sensitive to sound of frequencies around 800 Hz, but are likely to hear well above 1000 Hz, up to 4000 Hz (Higgs et al. 2002; Bretschneider et al. 2013). Furthermore, relative sensitivities for particle motion and sound pressure are likely to complement each other, but vary spectrally with a bias to the low end for particle motion and to the high end for sound pressure (Schulz-Mirbach et al. 2012). Future, tests could explore whether sound bursts restricted to relatively low (< 500 Hz) or relatively high (> 1000 Hz) frequencies in the audible range of zebrafish yield differential response patterns with respect to weighting of SVL and SPL. However, it should be noted that in the current study we had calibration limitations with our vector sensor that would have to be solved, as we were only able to assess particle motion levels within a range of 50-1000Hz.

As a final point, we like to highlight the phenomenon of particle ellipticity as a potentially relevant acoustic feature for auditory perception and sound-induced disturbance and deterrence in fish. The predicted levels of ellipticity at the locations of startle responses in our experimental set-up were highly variable, dependent on both spatial location and frequency range. Although the mechanism for determining directionality is not well understood in any fish species, the capacity for fish to localize a sound source based on the particle motion component of sound fields was recently nicely illustrated by a study on female midshipman fish (*Porichthys notatus*) approaching a speaker playing back a conspecific male call (Zeddies et al. 2012). We expect that higher degrees of particle ellipticity will diminish a fish's ability to localize sound sources (c.f. Rollo & Higgs 2008), thus reporting measures of particle ellipticity and incorporating them into statistical analysis may be valuable for future studies.

Conclusions

Our findings highlight the importance of reporting particle motion measurements in sound impact studies on fish. This is especially important for indoor studies in fish tanks, as we have shown that particle motion and sound pressure components do not share the same relationship in small tanks as they would in open water, far-field conditions. Furthermore, our exploration of the link between detailed characteristics of the underwater sound field and behavioural response tendencies of captive zebrafish revealed that both components of sound may be independently correlated to anxiety-related behavior such as freezing. Whether particle motion (SVL), sound pressure (SPL) or the ratio between particle velocity and sound pressure (excess SVL) are more or less prominent perceptually and responsible for specific anxiety-related, sound-induced escape or freezing behavior requires further study.

The practical challenges for further study are numerous. The lack of standardized methodology, low repeatability, and difficulty in obtaining commercially available geophones and accelerometers still remain obstacles for researchers (Radford et al. 2012; Anderson 2013). The highly complex sound field conditions (Parvulescu 1967; Akamatsu et al. 2002; Slabbekoorn 2016) also remain an issue for indoor studies in fish tanks, as should be clear from our own study. Nevertheless, we advocate the exploitation of indoor and outdoor conditions as complementary studies. Furthermore, intensive collaboration among fish biologists, acoustic engineers, and behavioural specialists remains critical for further progress in our fundamental understanding of the acoustic world of both captive and free-ranging fish (e.g. Shafiei Sabet et al. 2016. Neo et al. In Press).

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Supplementary material

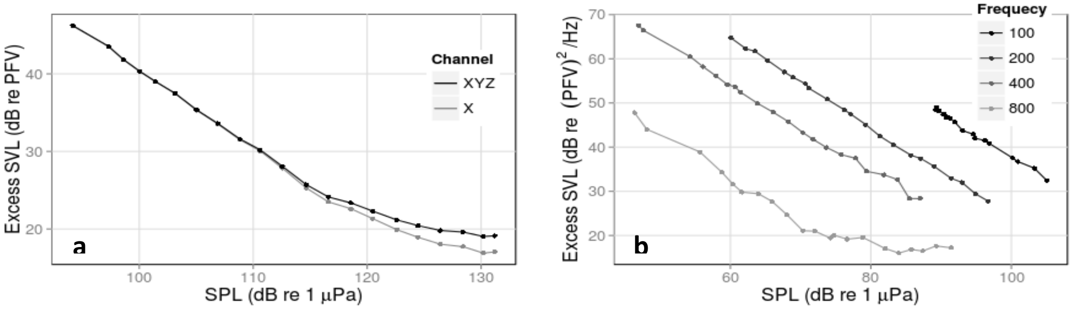

Fig. S1. The resulting excess SVL measurements from white noise playback with variable volume levels and a fixed vector sensor position: There is a negative relationship between excess SVL and SPL, independent of spatial location within the tank. The tank as ensonified with 21 white noise exposures, where each exposure was digitally adjusted to be 2dB quieter than the last. Fig. a) shows the excess particle velocity measured along the x axis (black), which is facing towards the speaker, and the summed particle velocity across all 3 channels of the vector sensor (grey). Fig. b) shows the excess particle velocity summed across all channels per selected frequency resulting from a PSD analysis (window length: 40000, window type: Hamming).

Table 1. Summary statistics for the Generalized Linear Model used to compare the spatial properties (i.e. the position of the vector sensor in the tank) and SPL of each measurement to the resulting Excess SVL. Coefficients are reported in cm, except for ordinal polynomials which are reported in transformed units to avoid the effects of covariance.

Excess SVL

Effect	**Coefficient**	***t*-value**
Intercept	159	49.6***
SPL	-1.17	59.6***
Distance from the wall closest to the in-air speaker	0.29	7.85***
Distance from a wall facing the direction adjacent to that of sound propagation: *1st degree polynomial*	-4.68	-1.91.
Distance from a wall facing the direction adjacent to that of sound propagation: *2nd degree polynomial*	-8.41	-3.43**
Distance from tank bottom	-0.189	-4.26***
Close to either wall facing the direction of sound propagation: *True*	2.17	3.37**
Close to either the bottom of the tank or the water surface: *True*	-3.05	-3.84***
Interaction: (Distance from a wall facing the direction adjacent to that of sound propagation: *2nd degree polynomial*)*(Distance from tank bottom)	0.350	3.52***
Interaction: (Distance from the wall closest to the in-air speaker)*(Distance from tank bottom)	-1.48e-2	-9.81***
Interaction: (Close to either wall facing the direction of sound propagation: *True*)*(Distance from tank bottom)	0.257	9.77***
Interaction: (Close to either wall facing the direction of sound propagation: *True*)*(Close to either the bottom of the tank or the water surface: *True*)	1.34	2.83**
Interaction: (Distance from the wall closest to the in-air speaker)*(Close to either the bottom of the tank or the water surface: *True*)	0.145	5.60***

Null Deviance: 5252.279 (n = 63); **Residual Deviance: 30.553** (n = 48)

*** p-value < 0.001; * p-value < 0.05; . p-value < 0.1

Table 2. Summary statistics for the Mixed Models comparing average swimming speed before and after noise exposure and the probability of a freeze response to SPL and SVL.

Post-exposure Average Swimming Speed			**Probability of Post-exposure Freeze response**		
Fixed effect	**Coefficient**	***t*-value**	**Fixed effect**	**Coefficient**	***z*-value**
Intercept	11.59	5.39***	***Intercept***	-22.71	-2.22**
Exposure Number	-0.51	-2.60*	SVL	-0.29	-2.45*
Average swimming speed before exposure	0.48	1.87**.**	Average swimming speed before exposure	-0.64	-2.61*
Expression of freezing behavior before exposure	-2.57	-2.13*			
	R^2_m	**R^2_c**		**R^2_m**	**R^2_c**
	0.15	0.43		0.32	0.79

*** p-value < 0.001; * p-value < 0.05; . p-value < 0.1

Table 3. Excess SVL values calculated from outdoor studies.

Excess SVL (dB ref 1nm/s)	**Sound Source**	**Bandwidth (Hz)**	**Reference**
11.8	Ambient Conditions	10-10000	Farina & Armelloni (2012)
10.9	Passing Boat	10-10000	-
-1.5	Ambient Conditions	200-2000	Neo et al. (In Press)
6	White noise from underwater speaker	200-2000	-